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Remarks:

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listing and declared, that it includes no new matter.

(54) Polyester synthase gene and process for producing polyester

(57) The present invention relates to a polyester synthase gene coding for a polypeptide containing the amino acid sequence of SEQ ID NO:2 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about polyester synthase activity; a gene expression cassette comprising the polyester synthase gene and either of open reading frames located upstream and downstream of said gene; a recombinant vector comprising the gene expression cassette; a transformant transformed with the recombinant vector; and a process for producing polyester by culturing the transformant in a medium and recovering polyester from the resulting culture.

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Description

Field of the Invention

5 The present invention relates to a polyester synthase gene, a recombinant vector containing the gene, a transformant carrying the recombinant vector, and a process for producing polyester by use of the transformant.

Background of the Invention

10 It is known that a large number of microorganisms biosynthesize poly-3-hydroxybutyrate (P(3HB)) and store it in the form of ultrafine particles as an energy source in the body. P(3HB) extracted from microorganisms is a thermoplastic polymer with a melting temperature of about 180 °C, and because of its excellent biodegradability and biocompatibility it is drawing attention as "green" plastic for preservation of the environment. Further, P(3HB) is "green" plastic which can be synthesized from regenerable carbon resources including sugars and vegetable oils by various microorganisms.
 15 However, P(3HB) is a highly crystalline polymer and thus has the problem in physical properties of inferior resistance to impact, so its practical application has never been attempted.

Recently, polyester P(3HB-co-3HH) as a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HH) and a process for producing the same have been studied and developed, and these are described in e.g. Japanese Patent Laid Open Publication Nos. 93049/1993 and 265065/1995 respectively. In these publications, the P(3HB-co-3HH) copolymer is produced from alkanolic acids or olive oil by fermentation with Aeromonas caviae isolated from soil. It is revealed that because the degree of crystallinity of the P(3HB-co-3HH) copolymer produced through fermentation is reduced with an increasing ratio of the 3HH unit in it, so that the copolymer becomes a soft polymeric material excellent in thermostability and formability and can be manufactured into strong yarn or transparent flexible film (Y. Doi, S. Kitamura, H. Abe, *Macromolecules* **28**, 4822-4823 (1995)). However, the yield of polyester (content of polyester in dried microorganisms) according to the processes described in Japanese Patent Laid Open Publication Nos. 93049/1993 and 265065/1995 is low, and thus there is demand for developments in a process for producing the copolymerized polyester P(3HB-co-3HH).
 25

Summary of the Invention

30 The object of the present invention is to provide a polyester synthase gene, recombinant vectors containing the gene, transformants transformed with the recombinant vectors, and processes for producing polyester by use of the transformants.

As a result of their eager research, the present inventors succeeded in producing the polyester in high yield by cloning a polyester synthase gene and deleting one or both of open reading frames located upstream and downstream of said gene to arrive at the completion of the present invention.
 35

That is, the present invention is a polyester synthase gene coding for a polypeptide containing the amino acid sequence of SEQ ID NO:2 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about polyester synthase activity. Said gene includes those containing e.g. the nucleotide sequence of SEQ ID NO:1.
 40

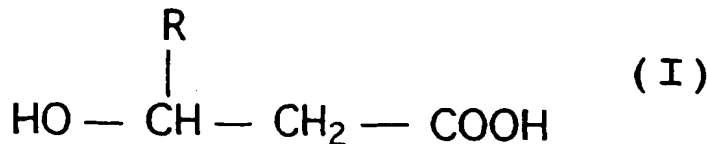
Further, the present invention is a gene expression cassette comprising said polyester synthase gene and either of open reading frames located upstream and downstream of said gene. In said gene expression cassette, the open reading frame located upstream of the polyester synthase gene includes those (e.g. SEQ ID NO:3) containing DNA coding for the amino acid sequence of SEQ ID NO:4, and the open reading frame located downstream of the polyester synthase gene includes those (e.g. SEQ ID NO:5) containing DNA coding for a polypeptide containing the amino acid sequence of SEQ ID NO:6 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about enoyl-CoA hydratase activity.
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Even if one or more amino acids in the amino acid sequence of SEQ ID NO:2 have undergone mutations such as deletion, replacement, addition etc., DNA coding for a polypeptide containing said amino acid sequence is also contained in the gene of the present invention insofar as the polypeptide has polyester synthase activity. For example, DNA coding for the amino acid sequence of SEQ ID NO:2 where methionine at the first position is deleted is also contained in the gene of the present invention.
 50

Further, the present invention is recombinant vectors comprising said polyester synthase gene or said gene expression cassette.

55 Further, the present invention is transformants transformed with said recombinant vectors.

Further, the present invention is processes for producing polyester, wherein said transformant is cultured in a medium, and polyester is recovered from the resulting culture. Examples of such polyester are copolymers (e.g. poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) random copolymers) of 3-hydroxyalkanoic acid represented by formula I:



wherein R represents a hydrogen atom or a C1 to C4 alkyl group.

Brief Description of the Drawing

FIG. 1 shows the structure of the gene of the present invention.

FIG. 2 is a photograph showing the result of SDS-polyacrylamide gel electrophoresis.

Detailed Description of the Invention

Hereinafter, the present invention is described in detail.

(1) Cloning of Polyester synthase gene

The polyester synthase gene of the present invention is separated from a microorganism belonging to the genus Aeromonas.

First, genomic DNA is isolated from a strain having the polyester synthase gene. Such a strain includes e.g. Aeromonas caviae.

Any known methods can be used for preparation of genomic DNA. For example, Aeromonas caviae is cultured in LB medium and then its genomic DNA is prepared by the hexadecyl trimethyl ammonium bromide method (Current Protocols in Molecular Biology, vol. 1, page 2.4.3., John Wiley & Sons Inc., 1994).

The DNA obtained in this manner is partially digested with a suitable restriction enzyme (e.g. Sau3AI, BamHI, BglII etc.) and then the DNA fragments are then dephosphorylated by treatment with alkaline phosphatase. It is ligated into a vector previously cleaved with a restriction enzyme (e.g. BamHI, BglII etc.) to prepare a library.

Phage or plasmid capable of autonomously replicating in host microorganisms is used as the vector. The phage vector includes e.g. EMBL3, M13, λ gt11 etc., and the plasmid vector includes e.g. pBR322, pUC18, and pBluescript II (Stratagene). Vectors capable of autonomously replicating in 2 or more host cells such as E. coli and Bacillus brevis, as well as various shuttle vectors, can also be used. Such vectors are also cleaved with said restriction enzymes so that their fragment can be obtained.

Conventional DNA ligase is used to ligate the resulting DNA fragments into the vector fragment. The DNA fragments and the vector fragment are annealed and then ligated to produce a recombinant vector.

To introduce the recombinant vector into a host microorganism, any known methods can be used. For example, if the host microorganism is E. coli, the calcium method (Lederberg, E.M. et al., J. Bacteriol. 119, 1072 (1974)) and the electroporation method (Current Protocols in Molecular Biology, vol. 1, page 1.8.4 (1994)) can be used. If phage DNA is used, the in vitro packaging method (Current Protocols in Molecular Biology, vol. 1, page 5.7.1 (1994)) etc. can be adopted. In the present invention, an in vitro packaging kit (Gigapack II, produced by Stratagene etc.) can also be used.

To obtain a DNA fragment containing the polyester synthase gene derived from Aeromonas caviae, a probe is then prepared. The amino acid sequences of some polyester synthase have already been known (Peoples, O.P. and Sinskey, A.J., J. Biol. Chem., 264, 15293 (1989); Huisman, G.W. et al., J. Biol. Chem., 266, 2191 (1991); Pieper, U. et al., FEMS Microbiol. Lett., 96, 73 (1992) etc.). Two conserved regions are selected from these amino acid sequences, and nucleotide sequences coding them are estimated to design oligonucleotides for use as primers. Examples of such oligonucleotides include, but are not limited to, the 2 oligonucleotides 5'-CC(C/G)CC(C/G)TGGATCAA(T/C)AAGT(T/A)(T/C)TA(T/C)ATC-3' (SEQ ID NO:7) and 5'-(G/C)AGCCA (G/C)GC(G/C)GTCCA(A/G)TC(G/C)GGCCACCA-3' (SEQ ID NO:8).

Polymerase chain reaction (PCR) (Molecular Cloning, vol. 2, page 14.2 (1989)) is carried out using these oligonucleotides as primers and the genomic DNA of Aeromonas caviae as a template. The partial fragment of polyester synthase gene is amplified by PCR.

Then, the partially amplified fragment thus obtained is labeled with a suitable reagent and used for colony hybridization of the above genomic DNA library (Current Protocols in Molecular Biology, vol. 1, page 6.0.3 (1994)).

The E. coli is screened by colony hybridization, and a plasmid is recovered from it using the alkaline method (Cur-

rent Protocols in Molecular Biology, vol. 1, page 1.6.1 (1994)), whereby a DNA fragment containing the polyester synthase gene is obtained.

The nucleotide sequence of said DNA fragment can be determined in e.g. an automatic nucleotide sequence analyzer such as 373A DNA sequencer (Applied Biosystems) using a known method such as the Sanger method (Molecular Cloning, vol. 2, page 13.3 (1989)).

The nucleotide sequence of the polyester synthase gene of the present invention is shown in SEQ ID NO:1, and the amino acid sequence encoded by said gene is shown in SEQ ID NO:2, where some amino acids may have undergone mutations such as deletion, replacement, addition etc. insofar as a polypeptide having said amino acid sequence brings about polyester synthase activity. Further, the gene of the present invention encompasses not only the nucleotide sequence coding for the amino acid sequence of SEQ ID NO:2 but also its degenerated isomers which except for degeneracy codons, code for the same polypeptide.

The above mutations such as deletion etc. can be induced by known site-directed mutagenesis (Current Protocols in Molecular Biology, vol. 1, page 8.1.1 (1994)).

After the nucleotide sequence was determined by the means described above, the gene of the present invention can be obtained by chemical synthesis or the PCR technique using genomic DNA as a template, or by hybridization using a DNA fragment having said nucleotide sequence as a probe.

(2) Preparation of Transformant

The transformant of the present invention is obtained by introducing the recombinant vector of the present invention into a host compatible with the expression vector used in constructing said recombinant vector.

The host is not particularly limited insofar as it can express the target gene. Examples are bacteria such as microorganisms belonging to the genus Alcaligenes, microorganisms belonging to the genus Pseudomonas, microorganisms belonging to the genus Bacillus, yeasts such as the genera Saccharomyces, Candida etc., and animal cells such as COS cells, CHO cells etc.

If bacteria such as microorganisms belonging to the genus Alcaligenes, microorganisms belonging to the genus Pseudomonas etc. are used as the host, the recombinant DNA of the present invention is preferably constituted such that it contains a promoter, the DNA of the present invention, and a transcription termination sequence so as to be capable of autonomous replication in the host. The expression vector includes pLA2917 (ATCC 37355) containing replication origin RK2 and pJRD215 (ATCC 37533) containing replication origin RSF1010, which are replicated and maintained in a broad range of hosts.

The promoter may be any one if it can be expressed in the host. Examples are promoters derived from E. coli, phage etc., such as trp promoter, lac promoter, P_L promoter, P_R promoter and T7 promoter. The method of introducing the recombinant DNA into bacteria includes e.g. a method using calcium ions (Current Protocols in Molecular Biology, vol. 1, page 1.8.1 (1994)) and the electroporation method (Current Protocols in Molecular Biology, vol. 1, page 1.8.4 (1994)).

If yeast is used as the host, expression vectors such as YEpl3, YCp50 etc. are used. The promoter includes e.g. gal 1 promoter, gal 10 promoter etc. To method of introducing the recombinant DNA into yeast includes e.g. the electroporation method (Methods. Enzymol., 194, 182-187 (1990)), the spheroplast method (Proc. Natl. Acad. Sci. USA, 84, 1929-1933 (1978)), the lithium acetate method (J. Bacteriol., 153, 163-168 (1983)) etc.

If animal cells are used as the host, expression vectors such as pcDNA1, pcDNA1/Amp (produced by Invitrogene) etc. are used. The method of introducing the recombinant DNA into animal cells includes e.g. the electroporation method, potassium phosphate method etc.

The nucleotide sequence determined as described above contains the polyester synthase gene as well as a plurality of open reading frames (ORFs) upstream and downstream of it. That is, the polyester synthase gene forms an operon with at least 2 ORF's under the control of a single promoter region.

The ORF's which are located respectively upstream and downstream of the polyester synthase gene are referred to hereinafter as "ORF1" and "ORF3".

It is considered that ORF1 is an open reading frame of a gene involved in accumulating polyester in the microorganism or a gene in the polyester biosynthesis system. It was revealed that ORF3 is an open reading frame of a gene coding for enoyl-CoA hydratase (particularly (R)-specific enoyl-CoA hydratase) involved in biosynthesis of polyester.

As shown in FIG. 1, an EcoRI fragment carrying an expression regulatory region (expressed as "-35/-10" in FIG. 1A), the polyester synthase gene, ORF1, and ORF3 was cloned in the present invention (FIG. 1A). This fragment is designated EE32.

Then, a fragment (a gene expression cassette) is prepared by deleting ORF1 and/or ORF3 from EE32, and this cassette is introduced into a host whereby a transformant capable of efficiently producing polyester can be obtained.

In EE32, a restriction enzyme BglII sites are introduced into regions between the expression regulatory region and the translation initiation codon of ORF1 and between the translation termination codon of ORF1 and the translation ini-

tiation codon of the polyester synthase gene, and then ORF1 is deleted from EE32 by treatment with BglII (FIG. 1B). Similarly, a restriction enzyme BamHI sites is introduced into a region between the translation termination codon of the polyester synthase gene and ORF3, and then ORF3 is deleted by treatment with BamHI (FIG. 1C).

To delete both ORF1 and ORF3, EE32 may be subjected to the above operation of deleting ORF1 and ORF3 (FIG. 1D).

The restriction enzyme sites can be introduced by site-directed mutagenesis using synthetic oligonucleotides (Current Protocols in Molecular Biology, vol. 1, page 8.1.1 (1994)).

Each gene expression cassette thus obtained is inserted into said plasmid capable of expression (e.g. pJRD215 (ATCC 37533)) and the resulting recombinant vector is used to transform *Alcaligenes eutrophus* PHB-4 (DSM541) (strain deficient in the ability to synthesize polyester). The method for this transformation includes e.g. the calcium chloride method, rubidium chloride method, low pH method, *in vitro* packaging method, conjugation transfer method etc.

(3) Production of Polyester

The production of polyester is carried out by culturing the transformant of the present invention in a medium, forming and accumulating the polyester of the present invention in the microorganism or in the culture, and recovering the polyester from the cultured microorganism or from the culture.

A conventional method used for culturing the host is also used to culture the transformant of the present invention.

The medium for the transformant prepared from a microorganism belonging to the genus *Alcaligenes* or *Pseudomonas* as the host include a medium containing a carbon source assimilable by the microorganism, in which a nitrogen source, inorganic salts or another organic nutrition source has been limited, for example a medium in which the nutrition source has been limited to 0.01 to 0.1 %.

The carbon source is necessary for growth of the microorganism, and it is simultaneously a starting material of polyester. Examples are hydrocarbons such as glucose, fructose, sucrose, maltose etc. Further, fat and oil related substances having 2 or more carbon atoms can be used as the carbon source. The fat and oil related substances include natural fats and oils, such as corn oil, soybean oil, safflower oil, sunflower oil, olive oil, coconut oil, palm oil, rape oil, fish oil, whale oil, porcine oil and cattle oil, aliphatic acids such as acetic acid, propionic acid, butanoic acid, pentanoic acid, hexoic acid, octanoic acid, decanoic acid, lauric acid, oleic acid, palmitic acid, linolenic acid, linolic acid and myristic acid as well as esters thereof, alcohols such as ethanol, propanol, butanol, pentanol, hexanol, octanol, lauryl alcohol, oleyl alcohol and palmityl alcohol as well as esters thereof.

The nitrogen source includes e.g. ammonia, ammonium salts such as ammonium chloride, ammonium sulfate, ammonium phosphate etc., peptone, meat extract, yeast extract, corn steep liquor etc. The inorganic matter includes e.g. monopotassium phosphate, dipotassium phosphate, magnesium phosphate, magnesium sulfate, sodium chloride etc.

Culture is carried out usually under aerobic conditions with shaking at 25 to 37 °C for more than 24 hours (e.g. 1 to 7 days) after expression is induced. During culture, antibiotics such as ampicillin, kanamycin, antipyrine, tetracycline etc. may be added to the culture. Polyester is accumulated in the microorganism by culturing it, and the polyester is then recovered.

To culture the microorganism transformed with the expression vector using an inducible promoter, its inducer can also be added to the medium. For example, isopropyl-β-D-thiogalactopyranoside (IPTG), indoleacrylic acid (IAA) etc. can be added to the medium.

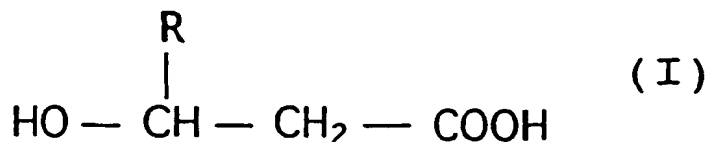
To culture the transformant from animal cells as the host, use is made of a medium such as RPMI-1640 or DMEM which may be supplemented with fetal bovine serum. Culture is carried out usually in the presence of 5 % CO₂ at 30 to 37°C for 14 to 28 days. During culture, antibiotics such as kanamycin, penicillin etc. may be added to the medium.

In the present invention, purification of polyester can be carried out e.g. as follows:

The transformant is recovered from the culture by centrifugation, then washed with distilled water and dried. Thereafter, the dried transformant is suspended in chloroform and heated to extract polyester from it. The residues are removed by filtration. Methanol is added to this chloroform solution to precipitate polyester. After the supernatant is removed by filtration or centrifugation, the precipitates are dried to give purified polyester.

The resulting polyester is confirmed to be the desired one in a usual manner e.g. by gas chromatography, nuclear magnetic resonance etc.

The gene of the present invention contains the polyester synthase gene isolated from *Aeromonas caviae*. This synthase can synthesize a copolymer (polyester) consisting of a monomer unit 3-hydroxyalkanoic acid represented by formula I:



wherein R represents a hydrogen atom or a C1 to C4 alkyl group. Said copolymer includes e.g. poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) random copolymer (P(3HB-co-3HH)) etc. and the transformant carrying said polyester synthase gene has the ability to produce P(3HB-co-3HH) with very high efficiency.

Conventionally, a process for producing poly-3-hydroxybutyrate (P(3HB)) or poly(3-hydroxybutyrate-co-3-hydroxyvalerate) random copolymer P(3HB-co-3HV) has been studied and developed, but such polyester has the problem in physical properties of inferior resistance to impact because it is a highly crystalline polymer.

Because degree of crystallinity is lowered by introducing 3-hydroxyhexanoate having 6 carbon atoms into a polymer chain, polyester acts as a flexible polymeric material which is also excellent in thermostability and formability, but conventional processes for producing P(3HB-co-3HH) by use of *Aeromonas caviae* (Japanese Patent Laid Open Publication Nos. 93049/1993 and 265065/1995) suffer from a low yield of polyester.

In the present invention, the P(3HB-co-3HH) copolyester can be produced in high yield.

Because the desired polyester can be obtained in a large amount using the above means, it can be used as a biodegradable material of yarn or film, various vessels etc. Further, the gene of the present invention can be used to breed a strain highly producing the P(3HB-co-3HH) copolymer polyester.

Examples

Hereinafter, the present invention is described in more detail with reference to the Examples which however are not intended to limit the scope of the present invention. (Example 1] Cloning of the Polyester synthase Gene from *Aeromonas caviae*

First, a genomic DNA library was prepared from *Aeromonas caviae*.

Aeromonas caviae FA440 was cultured overnight in 100 ml LB medium (1 % yeast extract, 0.5 % trypton, 0.5 % sodium chloride, 0.1 % glucose, pH 7.5) at 30 °C and then genomic DNA was obtained from the microorganism using the hexadecyl trimethyl ammonium bromide method (Current Protocols in Molecular Biology, vol. 1, page 2.4.3 (1994), John Wiley & Sons Inc.).

The resulting genomic DNA was partially digested with restriction enzyme Sau3AI. The vector plasmid used was cosmid vector pLA2917 (ATCC 37355).

This plasmid was cleaved with restriction enzyme BglII and dephosphorylated (Molecular Cloning, vol. 1, page 5.7.2 (1989), Cold Spring Harbor Laboratory) and then ligated into the partially digested genomic DNA fragment by use of DNA ligase.

E. coli S17-1 was transformed with this ligated DNA fragment by the *in vitro* packaging method (Current Protocols in Molecular Biology, vol. 1, page 5.7.2 (1994)) whereby a genomic DNA library from *Aeromonas caviae* was obtained.

To obtain a DNA fragment containing the polyester synthase gene from *Aeromonas caviae*, a probe was then prepared. Two well conserved regions were selected from known amino acid sequences of several polyester synthases, and nucleotide sequences coding for them were estimated, and 2 oligonucleotides 5'-CC(C/G)CC(C/G)TGGATCAA(T/C)AAGT (T/A)(T/C) TA(T/C)ATC-3' (SEQ ID NO:7) and 5'-(G/C)AGCCA(G/C)GC(G/C)GTCCA(A/G)TC(G/C)GGCCACCA-3' (SEQ ID NO:8) were synthesized.

The polyester synthase gene was partially amplified by PCR using these oligonucleotides as primers and the genomic DNA from *Aeromonas caviae* as a template. PCR was carried out using 30 cycles, each consisting of reaction at 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 60 seconds.

Then, this partially amplified fragment was labeled with digoxigenin using a DIG DNA labeling kit (Boehringer Mannheim) and used as a probe.

Using the probe thus obtained, *E. coli* carrying a plasmid containing the polyester synthase gene was isolated by colony hybridization from the genomic DNA library from *Aeromonas caviae*. By recovering the plasmid from the *E. coli*, a DNA fragment containing the polyester synthase gene was obtained.

The nucleotide sequence of a 3.2 kbp BglII-EcoRI fragment from this fragment was determined by the Sanger method.

As a result, the nucleotide sequence of the 3.2 kb fragment as shown in SEQ ID NOs:9 or 10 was determined.

By further examining homology to this nucleotide sequence, the polyester synthase gene containing the nucleotide sequence (1785 bp) of SEQ ID NO:1 could be identified in this 3.2 kb nucleotide sequence.

It should be understood that insofar as the protein encoded by the polyester synthase gene of the present invention has the function of gene expression for polyester polymerization, the nucleotide sequence of said gene may have undergone mutations such as deletion, replacement, addition etc.

In a fragment having the nucleotide sequence of SEQ ID NO:9 or 10, a 405 bp gene (ORF3) and a transcription termination region located downstream of the above 1785 bp nucleotide sequence, as well as a 354 bp gene (ORF1) and an expression regulatory region located upstream thereof were identified. The nucleotide sequence of ORF1 is shown in SEQ ID NO:4; the nucleotide sequence of ORF3 in SEQ ID NO:5; and the amino acid sequence encoded by ORF3 in SEQ ID NO: 6.

ORF3 is an open reading frame of a gene coding for enoyl-CoA hydratase involved in biosynthesis of polyester. Insofar as a polypeptide having the amino acid sequence encoded by ORF3 has enoyl-CoA hydratase activity, particularly (R)-specific enoyl-CoA hydratase activity, said amino acid sequence may have undergone mutations such as deletion, replacement and addition of one or more amino acids.

In the nucleotide sequences of SEQ ID NOS:9 and 10, the expression regulatory region is located at the 1- to 383-positions and the transcription termination region at the 3010 to 3187- positions.

[Example 2] Preparation of *Alcaligenes eutrophus* Transformant

The BglII site of the BglII-EcoRI fragment containing this expression regulatory region, ORF1, the polyester synthase gene, ORF3, and the transcriptional termination region was made EcoRI-ended by use of an EcoRI linker whereby a 3.2 kb EcoRI-EcoRI fragment (EE32 fragment) was obtained. This fragment was inserted into plasmid pJRD215 (ATCC 37533) capable of expression in microorganisms belonging to the genus *Alcaligenes*, and the resulting recombinant plasmid was transformed into *Alcaligenes eutrophus* PHB-4 (DSM 541) (strain deficient in the ability to synthesize polyester) by the conjugation transfer method, as follows:

First, the recombinant plasmid was used to transform *E. coli* S17-1 by the calcium chloride method. The recombinant *E. coli* thus obtained and *Alcaligenes eutrophus* PHB-4 were cultured overnight in 1.5 ml LB medium at 30 °C, and the respective cultures, each 0.1 ml, were combined and cultured at 30 °C for 4 hours. This microbial mixture was plated on MBF agar medium (0.9 % disodium phosphate, 0.15 % monopotassium phosphate, 0.05 % ammonium chloride, 0.5 % fructose, 1.5 % agar, 0.3 mg/ml kanamycin) and cultured at 30 °C for 5 days.

Because *Alcaligenes eutrophus* PHB-4 is rendered resistant to kanamycin by transferring the plasmid in the recombinant *E. coli* into it, the colonies grown on the MBF agar medium are a transformant of *Alcaligenes eutrophus*. One colony was isolated from these colonies so that *Alcaligenes eutrophus* AC32 (referred to hereinafter as AC32) was obtained.

AC32 has been deposited as FERM BP-6038 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan.

A restriction enzyme BglII sites were introduced respectively into regions upstream and downstream of the ORF1 gene in the EE32 fragment by site-directed mutagenesis using a synthetic oligonucleotide (Current Protocols in Molecular Biology, vol. 1, page 8.1.1 (1994)), and an ORF1 gene-free fragment was obtained by deleting the BglII-BglII fragment from the EE32 fragment and then inserted into plasmid pJRD215. The resulting recombinant plasmid was used to transform *Alcaligenes eutrophus* PHB-4 by the conjugation transfer method described above. The resulting transformant is referred to hereinafter as AC321.

Similarly, a restriction enzyme BamHI sites were introduced respectively regions upstream and downstream of the ORF3 gene in the EE32 fragment by site-directed mutagenesis, and an ORF3 gene-free fragment was obtained by deleting the BamHI-BamHI fragment from the EE32 fragment and then inserted into plasmid pJRD215. The resulting recombinant plasmid was used to transform *Alcaligenes eutrophus* PHB-4 by the conjugation transfer method described above. The resulting transformant is referred to hereinafter as AC323.

Similarly, a restriction enzyme BglII sites were introduced respectively regions upstream and downstream of the ORF1 gene and a restriction enzyme BamHI sites were introduced respectively regions upstream and downstream of the ORF3 gene in the EE32 fragment, and a gene fragment free of both the ORF1 and ORF3 genes was obtained by deleting the BglII-BglII and BamHI-BamHI fragments from the EE32 fragment and then inserted into plasmid pJRD215. The resulting recombinant plasmid was used to transform *Alcaligenes eutrophus* PHB-4 by the conjugation transfer method described above. The resulting transformant is referred to hereinafter as AC3213.

Further, the polyester synthase gene was amplified by PCR using the EE32 fragment as a template, and the resulting amplification product was inserted into a region between an expression regulatory region and a transcription termination region in a known polyester biosynthesis operon derived from *Alcaligenes eutrophus*. PCR was carried out using 5'-AGTTCGCCGCTCGGGTGTGGGTGAA-3' (SEQ ID NO: 11) and 5'-GGCATATGCGCTCATGCGGCGTCCT-3' (SEQ ID NO: 12) as primers in 30 cycles each consisting of reaction at 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 60 seconds.

This DNA fragment was inserted into plasmid pJRD215, and the resulting plasmid was used to transform *Alcali-*

genes *eutrophus* PHB-4 by the conjugation transfer method described above. The resulting transformant is referred to hereinafter as AC29.

[Example 3] Synthesis of Polyester by *Alcaligenes eutrophus* Transformants

Each of *Alcaligenes eutrophus* H16, PHB-4, AC32, AC321, AC323, AC3213 and AC29 was inoculated into 95 ml MB medium (0.9 % disodium phosphate, 0.15 % monopotassium phosphate, 0.05 % ammonium chloride) containing 1 ml of 1 % sodium octanoate and incubated in a flask at 30 °C. 0.2 g/L kanamycin was contained in the mediums for strains AC32, AC321, AC323, AC3213 and AC29. 12, 24, 36 and 48 hours thereafter, 1 ml of 1 % sodium octanoate was added to each medium (total amount of sodium octanoate added: 0.5 g) and the microorganisms were cultured for 72 hours.

Each of strains H16 and AC3213 was inoculated into the above MB medium to which 1% olive oil, palm oil, corn oil or oleic acid had been added, and each strain was cultured at 30 °C for 72 hours in a flask. 0.2 g/L kanamycin was contained in the mediums for strain AC3213.

Each of strains H16, AC32, AC321, AC323 and AC3213 was inoculated into the above MB medium to which 1% sodium heptanoate had been added, and each strain was cultured at 30 °C in a flask. 0.2 g/L kanamycin was contained in the mediums for strains AC32, AC321, AC323 and AC3213.

While 1 ml of 1% sodium heptanoate was added to each medium (total amount of sodium heptanoate added: 0.5 g) 12, 24, 36 and 48 hours thereafter, the microorganisms were cultured for 72 hours.

The microorganisms were recovered by centrifugation, washed with distilled water and lyophilized, and the weight of the dried microorganisms was determined. 2 ml sulfuric acid/methanol mixture (15 : 85) and 2 ml chloroform were added to 10-30 mg of the dried microorganism, and the sample was sealed and heated at 100 °C for 140 minutes whereby the polyester in the microorganisms was decomposed into methylester. 1 ml distilled water was added thereto and stirred vigorously. It was left and separated into 2 layers, and the lower organic layer was removed and analyzed for its components by capillary gas chromatography through a capillary column Neutra BOND-1 (column of 25 m in length, 0.25 mm in inner diameter and 0.4 µm in liquid film thickness, manufactured by GL Science) in Shimadzu GC-14A. The temperature was raised at a rate of 8 °C/min. from an initial temperature of 100 °C. The results are shown in Tables 1, 2 and 3.

Table 1

Synthesis of Polyester Using Octanoic Acid as Carbon Source				
Strain Used A. <i>eutrophus</i>	Weight of Dried Microorganism (g/l)	Content of Polyester in Dried Microorganism (weight-%)	Polyester Comp.	
			3HB	3HH
			(mole-%)	
H16	3.00	86	100	0
PHB-4	0.80	0	-	-
AC32	0.99	33	78	23
AC321	2.85	92	87	13
AC323	2.85	92	88	12
AC3213	3.64	96	85	15
AC29	3.20	94	92	8
3HB: 3-hydroxybutyrate, 3HH: 3-hydroxyhexanoate				

Table 2

Synthesis of Polyester Using Vegetable Oil or Oleic Acid as Carbon Source					
Strain Used <i>A. eutrophus</i>	Carbon Source	Weight of Dried Microorganism (g/l)	Content of Polyester in Dried Microorganism (weight-%)	Polyester Comp.	
				3HB	3HH
				(mole-%)	
H16	olive oil	4.27	79	100	0
	corn oil	3.57	81	100	0
	palm oil	4.13	79	100	0
	oleic acid	4.06	82	100	0
AC3213	olive oil	3.54	76	96	4
	corn oil	3.60	77	95	5
	palm oil	3.58	81	96	4
	oleic acid	2.22	70	96	4

3HB: 3-hydroxybutyrate, 3HH: 3-hydroxyhexanoate

Table 3

Synthesis of Polyester Using Heptanoic Acid as Carbon Source					
Strain Used <i>A. eutrophus</i>	Weight of Dried Microorganism (g/l)	Content of Polyester in Dried Microorganism (weight-%)	Polyester Comp.		
			3HB	3HV	3HHp
			(mole-%)		
H16	2.50	60	50	50	0
AC32	0.77	7	30	67	5
AC321	1.67	55	46	52	2
AC323	1.27	40	48	45	7
AC3213	2.76	67	44	48	8

3HB: 3-hydroxybutyrate, 3HH: 3-hydroxyhexanoate, 3HHp: 3-hydroxyheptanoate

As shown in Table 1, H16 (i.e. wild-type *Alcaligenes eutrophus*) synthesized a poly(3-hydroxybutyrate) homopolymer. This is because 3HH (3-hydroxyhexanoate) having 6 carbon atoms does not serve as a substrate for the polyester synthase possessed by H16. PHB-4 (i.e. the same strain as H16 but deficient in the ability to synthesize polyester) lacks the polyester synthase and thus does not accumulate polyester. AC32 prepared by introducing into PHB-4 the EE32 fragment containing the polyester synthase gene derived from *Aeromonas caviae* accumulated the poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) random copolymer (P(HB-co-3HH)) containing 22 mole-% 3HH (3-hydroxyhexanoate), and this copolymer accounted for 33 % by weight of the dried microorganism.

AC321, AC323 and AC3213 accumulated P(3HB-co-3HH) containing 12 to 15 mole-% 3HH, and the copolymer accounted for 92 to 96 % by weight of the dried microorganisms. As can be seen from these results, the ability of these strains to accumulate polyester was significantly improved by deleting the ORF1 gene and/or ORF3 gene.

P(3HB-co-3HH) was also accumulated in an amount of 94 % by weight of the microorganism even in the case of

AC29 carrying the polyester synthase gene derived from A. caviae whose expression regulatory region and transcriptional termination region had been replaced by those derived from Alcaligenes eutrophus, indicating that the yield of polyester was significantly improved even using the expression regulatory region and transcriptional termination region of different origin.

When AC3213 producing polyester in the highest yield was cultured using olive oil, corn oil or palm oil as a carbon source, the microorganism accumulated P(3HB-co-3HH) containing 4 to 5 mole-% 3HH, where the copolymer accounted for 76 to 81 % by weight of the microorganism, as shown in Table 2. Even if oleic acid as an fatty acid component contained most abundantly in vegetable oils was used as a carbon source, AC3213 accumulated P(3HB-co-3HH) containing 4 mole-% 3HH, where the copolymer accounted for 70 % by weight of the microorganism. Its corresponding wild strain H16 synthesized only poly(3-hydroxybutyrate) homopolymer under the same conditions.

Alcaligenes eutrophus FA440 is reported to have accumulated 8 % by weight of P(3HB-co-3HH) by use of palmitic acid as a carbon source (Japanese Patent Laid Open Publication No. 265065/1995). On the other hand, the transformant according to the present invention has accumulated 96 % by weight of P(3HB-co-3HH) by use of octanoic acid as a carbon source and 76 to 81 % by weight of P(3HB-co-3HH) by use of extremely cheap vegetable oils as a carbon source, so the comparison therebetween indicates that the method of synthesizing P(3HB-co-3HH) by the transformant used in the present example is an extremely superior method.

When heptanoic acid was used as a carbon source, H16, that is a wild strain of Alcaligenes eutrophus, synthesized poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer (P(3HB-co-3HV)). This is because 3HHp (3-hydroxyheptanoate) having 7 carbon atoms does not serve as a substrate for the polyester synthase possessed by H16, AC32, derived from PHB-4 by introduction of the EE32 fragment containing the polyester synthase gene derived from Aeromonas caviae, accumulated poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyheptanoate) terpolymer (P(3HB-co-3HV-co-3HHp)) containing 5 mole-% 3HHp, where this copolymer accounted for 7 % by weight of the dried microorganism.

Further, each of strains AC321, AC323 and AC3213 accumulated P(3HB-co-3HV-co-3HHp) containing 2 to 8 mole-% 3HHp where the copolymer accounted for 40 to 67 % by weight of the microorganisms, indicating that the yield of polyester was significantly improved by deleting the ORF1 gene and/or ORF3 gene (Table 3).

From these results, it is concluded that copolyesters consisting of 3-hydroxyalkanoic acid with 4 to 7 carbon atoms can be synthesized using the polyester synthase derived from Aeromonas caviae.

[Example 4] Identification of Functions of ORF3

The ORF3 gene was amplified by PCR using the EE32 fragment as a template and then inserted into a site downstream of T7 promoter in expression plasmid PET-3a (Novagene). PCR was carried out using 5'-GCCATATGAGCG-CACAATCCCTGGAAGTAG-3' (SEQ ID NO:13) and 5'-CTGGGATCCGCCGGTGCTTAAGGCAGCTTG-3' (SEQ ID NO:14) as primers in 25 cycles each consisting of reaction at 95 °C for 60 seconds and 68 °C for 30 seconds. The resulting plasmid was used to transform E. coli BL21 (DE3) (Novagene). The resulting transformant is designated NB3.

NB3 was cultured in LB medium at 30 °C for 4 hours, and isopropyl- β -D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.4 mM to induce expression, and it was further cultured at 30 °C for 2 hours. The microorganism was recovered by centrifugation, disrupted by ultrasonication and centrifuged to give a soluble protein fraction.

As shown in Table 4, high enoyl-CoA hydratase activity was detected in the soluble fraction from the microorganism having the expression plasmid introduced into it.

Table 4
Specific Activity of Enoyl-CoA Hydratase
in Soluble Protein Fraction

	(unit/mg protein)
<u>E. coli</u> BL21/PET-3a	0
<u>E. coli</u> NB3	1700

The enoyl-CoA hydratase activity was determined by measuring a change in absorbance (263 nm) due to double bond hydration, using crotonyl-CoA (Sigma) as substrate (concentration: 0.25 mM). No activity was detected in E. coli

into which the control plasmid PET-3a free of the ORF3 gene had been introduced.

Then, the enoyl-CoA hydratase protein was purified. A soluble protein fraction from NB3 was applied to an anion exchange column Q-Sepharose (Pharmacia) and eluted with a gradient of (0 to 1 M) NaCl, and a fraction with enoyl-CoA hydratase activity was collected. SDS-PAGE analysis indicated that the active fraction was homogenous in electrophoresis as shown in FIG. 2. In addition, about 3-fold specific activity could be attained as shown in Table 5.

Table 5

Specific Activity of Enoyl-CoA Hydratase

	(unit/mg protein)
E. coli NB3 soluble protein fraction	1700
<u>anion exchange column elution fraction</u>	<u>5100</u>

The N-terminal amino acid sequence of the enoyl-CoA hydratase protein thus purified was determined. As shown in Table 6, the determined amino acid sequence was the same except for Met in the initiation codon as the amino acid sequence deduced from the nucleotide sequence of the ORF3 gene.

Table 6

Comparison between Amino Acid Sequences

	(unit/mg protein)
N-terminal amino acid sequence of	
purified enoyl-CoA hydratase: SAQSLEVGQKARLSKRFGAA (SEQ ID NO:15)	
amino acid sequence deduced from	
<u>ORF3 nucleotide sequence: MSAQSLEVGQKARLSKRFGAA (SEQ ID NO:16)</u>	

From this, it could be confirmed that ORF3 codes for enoyl-CoA hydratase. It is considered that Met was released by post-translational modification.

Further, the stereospecificity of enoyl-CoA hydratase encoded by ORF3 was examined as follows:

By adding (S)-3-hydroxybutyryl-CoA dehydrogenase (Sigma) (final concentration: 0.2 unit/ml) and oxidized nicotinamide adenine dinucleotide (NAD⁺) (final concentration: 0.5 mM) to a reaction solution for activity measurement, (S)-3-hydroxybutyryl-CoA formed is oxidized to acetoacetyl-CoA by the action of the dehydrogenase if the enoyl-CoA hydratase is specific to the (S)-isomer. During this reaction, NAD⁺ is reduced to form NADH resulting in the generation of a specific absorption at 340 nm. If enoyl-CoA hydratase is specific to the (R)-isomer, NADH is not formed.

As shown in Table 7, the change in absorbance at 340 nm when enoyl-CoA hydratase encoded by ORF3 was used, was the same as in the case where enoyl-CoA hydratase was absent, but if commercially available (S)-specific enoyl-CoA hydratase (Sigma) was used, a change in absorbance due to formation of NADH was observed.

Table 7

Change in Absorbance at 340 nm after 1 Min.	
no addition of enoyl-CoA hydratase	0.045
ORF3-derived enoyl-CoA hydratase	0.047
(S)-isomer specific enoyl-CoA hydratase (Sigma)	0.146

From this result, it was made evident that the purified enoyl-CoA hydratase is specific to the (R)-isomer. Thus, it was found that ORF3 codes for (R)-isomer specific enoyl-CoA hydratase.

According to the present invention, there are provided a polyester synthase, a recombinant vector carrying the gene, a transformant carrying the recombinant vector and a process for producing polyester by use of the transformant.

The present invention is extremely useful in that the present gene codes for a polyester synthase capable of synthesizing polyester as a copolymer consisting of a monomer unit represented by 3-hydroxyalkanoic acid having 4 to 7 carbon atoms, and that the present process can synthesize a biodegradable plastic P(3HB-co-3HH) very efficiently which is excellent in thermostability and formability.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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 (B) STREET: Hirosawa 2-1
 (C) CITY: Wako-shi
 (D) STATE: Saitama
 (E) COUNTRY: Japan
 (F) POSTAL CODE (ZIP): 351-01
 (G) TELEPHONE: 81-48-467-9263
 (H) TELEFAX: 81-48-462-4609

(ii) TITLE OF INVENTION: POLYESTER SYNTHASE GENE AND PROCESS FOR PRODUCING POLYESTER

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: 97113932.4

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: JP 214509/1996
 (B) FILING DATE: 14-AUG-1996

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: JP 199979/1997
 (B) FILING DATE: 25-JUL-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1785 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 1..1782

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATG AGC CAA CCA TCT TAT GGC CCG CTG TTC GAG GCC CTG GCC CAC TAC	48
Met Ser Gln Pro Ser Tyr Gly Pro Leu Phe Glu Ala Leu Ala His Tyr	
1 5 10 15	
AAT GAC AAG CTG CTG GCC ATG GCC AAG GCC CAG ACA GAG CGC ACC GCC	96
Asn Asp Lys Leu Leu Ala Met Ala Lys Ala Gln Thr Glu Arg Thr Ala	
20 25 30	
CAG GCG CTG CTG CAG ACC AAT CTG GAC GAT CTG GGC CAG GTG CTG GAG	144
Gln Ala Leu Leu Gln Thr Asn Leu Asp Asp Leu Gly Gln Val Leu Glu	

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[illegible]

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	Gln	Leu	Ala	Val	Ser	Phe	Ser	Leu	Leu	Arg	Glu	Asn	Ser	Leu	Tyr	Trp	
	385					390					395					400	
	AAC	TAC	TAC	ATC	GAC	AGC	TAC	CTC	AAG	GGT	CAG	AGC	CCG	GTG	GCC	TTC	1248
5	Asn	Tyr	Tyr	Ile	Asp	Ser	Tyr	Leu	Lys	Gly	Gln	Ser	Pro	Val	Ala	Phe	
					405					410						415	
	GAT	CTG	CTG	CAC	TGG	AAC	AGC	GAC	AGC	ACC	AAT	GTG	GCG	GGC	AAG	ACC	1296
	Asp	Leu	Leu	His	Trp	Asn	Ser	Asp	Ser	Thr	Asn	Val	Ala	Gly	Lys	Thr	
					420					425						430	
	CAC	AAC	AGC	CTG	CTG	CGC	CGT	CTC	TAC	CTG	GAG	AAC	CAG	CTG	GTG	AAG	1344
10	His	Asn	Ser	Leu	Leu	Arg	Arg	Leu	Tyr	Leu	Glu	Asn	Gln	Leu	Val	Lys	
					435					440						445	
	GGG	GAG	CTC	AAG	ATC	CGC	AAC	ACC	CGC	ATC	GAT	CTC	GGC	AAG	GTG	AAG	1392
	Gly	Glu	Leu	Lys	Ile	Arg	Asn	Thr	Arg	Ile	Asp	Leu	Gly	Lys	Val	Lys	
					450					455						460	
	ACC	CCT	GTG	CTG	CTG	GTG	TCG	GCG	GTG	GAC	GAT	CAC	ATC	GCC	CTC	TGG	1440
	Thr	Pro	Val	Leu	Leu	Val	Ser	Ala	Val	Asp	Asp	His	Ile	Ala	Leu	Trp	
15					465											480	
	CAG	GGC	ACC	TGG	CAG	GGC	ATG	AAG	CTG	TTT	GGC	GGG	GAG	CAG	CGC	TTC	1488
	Gln	Gly	Thr	Trp	Gln	Gly	Met	Lys	Leu	Phe	Gly	Gly	Glu	Gln	Arg	Phe	
					485					490						495	
	CTC	CTG	GCG	GAG	TCC	GGC	CAC	ATC	GCC	GGC	ATC	ATC	AAC	CCG	CCG	GCC	1536
	Leu	Leu	Ala	Glu	Ser	Gly	His	Ile	Ala	Gly	Ile	Ile	Asn	Pro	Pro	Ala	
					500					505						510	
20	GCC	AAC	AAG	TAC	GGC	TTC	TGG	CAC	AAC	GGG	GCC	GAG	GCC	GAG	AGC	CCG	1584
	Ala	Asn	Lys	Tyr	Gly	Phe	Trp	His	Asn	Gly	Ala	Glu	Ala	Glu	Ser	Pro	
					515					520						525	
	GAG	AGC	TGG	CTG	GCA	GGG	GCG	ACG	CAC	CAG	GGC	GGC	TCC	TGG	TGG	CCC	1632
	Glu	Ser	Trp	Leu	Ala	Gly	Ala	Thr	His	Gln	Gly	Gly	Ser	Trp	Trp	Pro	
					530					535						540	
25	GAG	ATG	ATG	GGC	TTT	ATC	CAG	AAC	CGT	GAC	GAA	GGG	TCA	GAG	CCC	GTC	1680
	Glu	Met	Met	Gly	Phe	Ile	Gln	Asn	Arg	Asp	Glu	Gly	Ser	Glu	Pro	Val	
					545											560	
	CCC	GCG	CGG	GTC	CCG	GAG	GAA	GGG	CTG	GCC	CCC	GCC	CCC	GGC	CAC	TAT	1728
	Pro	Ala	Arg	Val	Pro	Glu	Glu	Gly	Leu	Ala	Pro	Ala	Pro	Gly	His	Tyr	
					565					570						575	
30	GTC	AAG	GTG	CGG	CTC	AAC	CCC	GTG	TTT	GCC	TGC	CCA	ACA	GAG	GAG	GAC	1776
	Val	Lys	Val	Arg	Leu	Asn	Pro	Val	Phe	Ala	Cys	Pro	Thr	Glu	Glu	Asp	
					580					585						590	
	GCC	GCA	TGA														1785
	Ala	Ala															

35 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 594 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

40 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

45	Met	Ser	Gln	Pro	Ser	Tyr	Gly	Pro	Leu	Phe	Glu	Ala	Leu	Ala	His	Tyr
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	Asn	Asp	Lys	Leu	Leu	Ala	Met	Ala	Lys	Ala	Gln	Thr	Glu	Arg	Thr	Ala
				20					25					30		
	Gln	Ala	Leu	Leu	Gln	Thr	Asn	Leu	Asp	Asp	Leu	Gly	Gln	Val	Leu	Glu
				35					40				45			
50	Gln	Gly	Ser	Gln	Gln	Pro	Trp	Gln	Leu	Ile	Gln	Ala	Gln	Met	Asn	Trp
		50					55					60				
	Trp	Gln	Asp	Gln	Leu	Lys	Leu	Met	Gln	His	Thr	Leu	Leu	Lys	Ser	Ala
	65					70					75					80

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	Gly	Gln	Pro	Ser	Glu	Pro	Val	Ile	Thr	Pro	Glu	Arg	Ser	Asp	Arg	Arg
					85					90					95	
	Phe	Lys	Ala	Glu	Ala	Trp	Ser	Glu	Gln	Pro	Ile	Tyr	Asp	Tyr	Leu	Lys
				100					105					110		
5	Gln	Ser	Tyr	Leu	Leu	Thr	Ala	Arg	His	Leu	Leu	Ala	Ser	Val	Asp	Ala
			115					120					125			
	Leu	Glu	Gly	Val	Pro	Gln	Lys	Ser	Arg	Glu	Arg	Leu	Arg	Phe	Phe	Thr
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	Arg	Gln	Tyr	Val	Asn	Ala	Met	Ala	Pro	Ser	Asn	Phe	Leu	Ala	Thr	Asn
		145			150						155				160	
10	Pro	Glu	Leu	Leu	Lys	Leu	Thr	Leu	Glu	Ser	Asp	Gly	Gln	Asn	Leu	Val
				165						170					175	
	Arg	Gly	Leu	Ala	Leu	Leu	Ala	Glu	Asp	Leu	Glu	Arg	Ser	Ala	Asp	Gln
				180					185					190		
	Leu	Asn	Ile	Arg	Leu	Thr	Asp	Glu	Ser	Ala	Phe	Glu	Leu	Gly	Arg	Asp
		195						200					205			
15	Leu	Ala	Leu	Thr	Pro	Gly	Arg	Val	Val	Gln	Arg	Thr	Glu	Leu	Tyr	Glu
		210				215						220				
	Leu	Ile	Gln	Tyr	Ser	Pro	Thr	Thr	Glu	Thr	Val	Gly	Lys	Thr	Pro	Val
		225			230						235				240	
	Leu	Ile	Val	Pro	Pro	Phe	Ile	Asn	Lys	Tyr	Tyr	Ile	Met	Asp	Met	Arg
				245					250					255		
20	Pro	Gln	Asn	Ser	Leu	Val	Ala	Trp	Leu	Val	Ala	Gln	Gly	Gln	Thr	Val
			260					265						270		
	Phe	Met	Ile	Ser	Trp	Arg	Asn	Pro	Gly	Val	Ala	Gln	Ala	Gln	Ile	Asp
		275					280						285			
	Leu	Asp	Asp	Tyr	Val	Val	Asp	Gly	Val	Ile	Ala	Ala	Leu	Asp	Gly	Val
		290				295					300					
25	Glu	Ala	Ala	Thr	Gly	Glu	Arg	Glu	Val	His	Gly	Ile	Gly	Tyr	Cys	Ile
		305			310					315					320	
	Gly	Gly	Thr	Ala	Leu	Ser	Leu	Ala	Met	Gly	Trp	Leu	Ala	Ala	Arg	Arg
				325						330					335	
	Gln	Lys	Gln	Arg	Val	Arg	Thr	Ala	Thr	Leu	Phe	Thr	Thr	Leu	Leu	Asp
			340					345						350		
30	Phe	Ser	Gln	Pro	Gly	Glu	Leu	Gly	Ile	Phe	Ile	His	Glu	Pro	Ile	Ile
			355				360						365			
	Ala	Ala	Leu	Glu	Ala	Gln	Asn	Glu	Ala	Lys	Gly	Ile	Met	Asp	Gly	Arg
		370				375						380				
	Gln	Leu	Ala	Val	Ser	Phe	Ser	Leu	Leu	Arg	Glu	Asn	Ser	Leu	Tyr	Trp
		385			390					395					400	
	Asn	Tyr	Tyr	Ile	Asp	Ser	Tyr	Leu	Lys	Gly	Gln	Ser	Pro	Val	Ala	Phe
			405							410				415		
35	Asp	Leu	Leu	His	Trp	Asn	Ser	Asp	Ser	Thr	Asn	Val	Ala	Gly	Lys	Thr
			420					425						430		
	His	Asn	Ser	Leu	Leu	Arg	Arg	Leu	Tyr	Leu	Glu	Asn	Gln	Leu	Val	Lys
		435					440					445				
	Gly	Glu	Leu	Lys	Ile	Arg	Asn	Thr	Arg	Ile	Asp	Leu	Gly	Lys	Val	Lys
		450				455					460					
40	Thr	Pro	Val	Leu	Leu	Val	Ser	Ala	Val	Asp	Asp	His	Ile	Ala	Leu	Trp
		465			470					475					480	
	Gln	Gly	Thr	Trp	Gln	Gly	Met	Lys	Leu	Phe	Gly	Gly	Glu	Gln	Arg	Phe
				485						490					495	
	Leu	Leu	Ala	Glu	Ser	Gly	His	Ile	Ala	Gly	Ile	Ile	Asn	Pro	Pro	Ala
			500					505						510		
45	Ala	Asn	Lys	Tyr	Gly	Phe	Trp	His	Asn	Gly	Ala	Glu	Ala	Glu	Ser	Pro
		515					520					525				
	Glu	Ser	Trp	Leu	Ala	Gly	Ala	Thr	His	Gln	Gly	Gly	Ser	Trp	Trp	Pro
		530				535						540				
	Glu	Met	Met	Gly	Phe	Ile	Gln	Asn	Arg	Asp	Glu	Gly	Ser	Glu	Pro	Val
		545			550					555					560	
50	Pro	Ala	Arg	Val	Pro	Glu	Glu	Gly	Leu	Ala	Pro	Ala	Pro	Gly	His	Tyr
				565						570				575		
	Val	Lys	Val	Arg	Leu	Asn	Pro	Val	Phe	Ala	Cys	Pro	Thr	Glu	Glu	Asp
			580					585						590		
	Ala	Ala														

55

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 354 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..351

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```

15  ATG ATG AAT ATG GAC GTG ATC AAG AGC TTT ACC GAG CAG ATG CAA GGC   u@48
    Met Met Asn Met Asp Val Ile Lys Ser Phe Thr Glu Gln Met Gln Gly
      1          5          10          15
20  TTC GCC GCC CCC CTC ACC CGC TAC AAC CAG CTG CTG GCC AGC AAC ATC   u@96
    Phe Ala Ala Pro Leu Thr Arg Tyr Asn Gln Leu Leu Ala Ser Asn Ile
      20          25          30
    GAA CAG CTG ACC CGG TTG CAG CTG GCC TCC GCC AAC GCC TAC GCC GAA   144
    Glu Gln Leu Thr Arg Leu Gln Leu Ala Ser Ala Asn Ala Tyr Ala Glu
      35          40          45
    CTG GGC CTC AAC CAG TTG CAG GCC GTG AGC AAG GTG CAG GAC ACC CAG   192
    Leu Gly Leu Asn Gln Leu Gln Ala Val Ser Lys Val Gln Asp Thr Gln
      50          55          60
25  AGC CTG GCG GCC CTG GGC ACA GTG CAA CTG GAG ACC GCC AGC CAG CTC   240
    Ser Leu Ala Ala Leu Gly Thr Val Gln Leu Glu Thr Ala Ser Gln Leu
      65          70          75          80
    TCC CGC CAG ATG CTG GAT GAC ATC CAG AAG CTG AGC GCC CTC GGC CAG   288
    Ser Arg Gln Met Leu Asp Asp Ile Gln Lys Leu Ser Ala Leu Gly Gln
      85          90          95
30  CAG TTC AAG GAA GAG CTG GAT GTC CTG ACC GCA GAC GGC ATC AAG AAA   336
    Gln Phe Lys Glu Glu Leu Asp Val Leu Thr Ala Asp Gly Ile Lys Lys
      100          105          110
    AGC ACG GGC AAG GCC TGA   354
    Ser Thr Gly Lys Ala
      115

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 117 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```

45  Met Met Asn Met Asp Val Ile Lys Ser Phe Thr Glu Gln Met Gln Gly
      1          5          10          15
    Phe Ala Ala Pro Leu Thr Arg Tyr Asn Gln Leu Leu Ala Ser Asn Ile
      20          25          30
    Glu Gln Leu Thr Arg Leu Gln Leu Ala Ser Ala Asn Ala Tyr Ala Glu
      35          40          45
50  Leu Gly Leu Asn Gln Leu Gln Ala Val Ser Lys Val Gln Asp Thr Gln
      50          55          60
    Ser Leu Ala Ala Leu Gly Thr Val Gln Leu Glu Thr Ala Ser Gln Leu

```

```

      65              70              75              80
Ser Arg Gln Met Leu Asp Asp Ile Gln Lys Leu Ser Ala Leu Gly Gln
      85              90              95
Gln Phe Lys Glu Glu Leu Asp Val Leu Thr Ala Asp Gly Ile Lys Lys
5      100              105              110
Ser Thr Gly Lys Ala
      115

```

2) INFORMATION FOR SEQ ID NO: 5:

```

10      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 405 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: double
          (D) TOPOLOGY: linear

```

```

15      (ii) MOLECULE TYPE: DNA (genomic)

```

```

      (ix) FEATURE:
          (A) NAME/KEY: CDS
          (B) LOCATION:1..402

```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```

      ATG AGC GCA CAA TCC CTG GAA GTA GGC CAG AAG GCC CGT CTC AGC AAG      48
      Met Ser Ala Gln Ser Leu Glu Val Gly Gln Lys Ala Arg Leu Ser Lys
      1              5              10              15
25      CGG TTC GGG GCG GCG GAG GTA GCC GCC TTC GCC GCG CTC TCG GAG GAC      96
      Arg Phe Gly Ala Ala Glu Val Ala Ala Phe Ala Ala Leu Ser Glu Asp
      20              25              30
      TTC AAC CCC CTG CAC CTG GAC CCG GCC TTC GCC GCC ACC ACG GCG TTC      144
      Phe Asn Pro Leu His Leu Asp Pro Ala Phe Ala Ala Thr Thr Ala Phe
      35              40              45
30      GAG CGG CCC ATA GTC CAC GGC ATG CTG CTC GCC AGC CTC TTC TCC GGG      192
      Glu Arg Pro Ile Val His Gly Met Leu Leu Ala Ser Leu Phe Ser Gly
      50              55              60
      CTG CTG GGC CAG CAG TTG CCG GGC AAG GGG AGC ATC TAT CTG GGT CAA      240
      Leu Leu Gly Gln Gln Leu Pro Gly Lys Gly Ser Ile Tyr Leu Gly Gln
      65              70              75              80
35      AGC CTC AGC TTC AAG CTG CCG GTC TTT GTC GGG GAC GAG GTG ACG GCC      288
      Ser Leu Ser Phe Lys Leu Pro Val Phe Val Gly Asp Glu Val Thr Ala
      85              90              95
      GAG GTG GAG GTG ACC GCC CTT CGC GAG GAC AAG CCC ATC GCC ACC CTG      336
      Glu Val Glu Val Thr Ala Leu Arg Glu Asp Lys Pro Ile Ala Thr Leu
      100              105              110
40      ACC ACC CGC ATC TTC ACC CAA GGC GGC GCC CTC GCC GTG ACG GGG GAA      384
      Thr Thr Arg Ile Phe Thr Gln Gly Gly Ala Leu Ala Val Thr Gly Glu
      115              120              125
      GCC GTG GTC AAG CTG CCT TAA      405
      Ala Val Val Lys Leu Pro
      130

```

(2) INFORMATION FOR SEQ ID NO: 6:

```

50      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 134 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS:
          (D) TOPOLOGY: linear

```

```

      (ii) MOLECULE TYPE: protein

```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

5 Met Ser Ala Gln Ser Leu Glu Val Gly Gln Lys Ala Arg Leu Ser Lys
 1 5 10 15
 Arg Phe Gly Ala Ala Glu Val Ala Ala Phe Ala Ala Leu Ser Glu Asp
 20 25 30
 Phe Asn Pro Leu His Leu Asp Pro Ala Phe Ala Ala Thr Thr Ala Phe
 35 40 45
 Glu Arg Pro Ile Val His Gly Met Leu Leu Ala Ser Leu Phe Ser Gly
 50 55 60
 10 Leu Leu Gly Gln Gln Leu Pro Gly Lys Gly Ser Ile Tyr Leu Gly Gln
 65 70 75 80
 Ser Leu Ser Phe Lys Leu Pro Val Phe Val Gly Asp Glu Val Thr Ala
 85 90 95
 Glu Val Glu Val Thr Ala Leu Arg Glu Asp Lys Pro Ile Ala Thr Leu
 100 105 110
 15 Thr Thr Arg Ile Phe Thr Gln Gly Ala Leu Ala Val Thr Gly Glu
 115 120 125
 Ala Val Val Lys Leu Pro
 130

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CCSCCSTGGA TCAAYAAGTW YTAYATC

27

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

SAGCCASGCS GTCCARTCSG GCCACCA

27

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3187 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

EP 0 824 148 A2

(A) NAME/KEY: CDS
(B) LOCATION: 384..734

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 830..2611

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

10	AGATCTGGAC CGGGGTGCTG GCCTGGGCCA CGCCGGCGAG GGCCAGCGCG GAGCAACCGA	60
	GCAGCAGGGC GAGAGGTTTC ATCGGGATTC CTTGGCAGTC TGAATGACGT GCCAGCCTAT	120
	CAGCGCGGCG CCGGTGCGGC GAGGGCGCGC CGGACCCAGT GCGTCACCTC TCGTCTGATC	180
	CGCCTCCCTC GACGGGCGTC GCTGACAAA AAAATCAAAC AGAAATTAAC ATTTATGTCA	240
	TTTACACCAA ACCGCATTG GTTGACGAAT GCTCAAACGT GTGTTTGAAC AGAGCAAGCA	300
	ACACGTAAAC AGGGATGACA TGCAGTACCC GTAAGAAGGG CCGATTGGCC CACAACAACA	360
	CTGTTCTGCC GAACTGGAGA CCG ATG ATG AAT ATG GAC GTG ATC AAG AGC	410
15	Met Met Asn Met Asp Val Ile Lys Ser	
	1 5	
	TTT ACC GAG CAG ATG CAA GGC TTC GCC GCC CCC CTC ACC CGC TAC AAC	458
	Phe Thr Glu Gln Met Gln Gly Phe Ala Ala Pro Leu Thr Arg Tyr Asn	
	10 15 20 25	
20	CAG CTG CTG GCC AGC AAC ATC GAA CAG CTG ACC CGG TTG CAG CTG GCC	506
	Gln Leu Leu Ala Ser Asn Ile Glu Gln Leu Thr Arg Leu Gln Leu Ala	
	30 35 40	
	TCC GCC AAC GCC TAC GCC GAA CTG GGC CTC AAC CAG TTG CAG GCC GTG	554
	Ser Ala Asn Ala Tyr Ala Glu Leu Gly Leu Asn Gln Leu Gln Ala Val	
	45 50 55	
	AGC AAG GTG CAG GAC ACC CAG AGC CTG GCG GCC CTG GGC ACA GTG CAA	602
	Ser Lys Val Gln Asp Thr Gln Ser Leu Ala Ala Leu Gly Thr Val Gln	
25	60 65 70	
	CTG GAG ACC GCC AGC CAG CTC TCC CGC CAG ATG CTG GAT GAC ATC CAG	650
	Leu Glu Thr Ala Ser Gln Leu Ser Arg Gln Met Leu Asp Asp Ile Gln	
	75 80 85	
	AAG CTG AGC GCC CTC GGC CAG CAG TTC AAG GAA GAG CTG GAT GTC CTG	698
	Lys Leu Ser Ala Leu Gly Gln Gln Phe Lys Glu Glu Leu Asp Val Leu	
30	90 95 100 105	
	ACC GCA GAC GGC ATC AAG AAA AGC ACG GGC AAG GCC TGATAACCCC	744
	Thr Ala Asp Gly Ile Lys Lys Ser Thr Gly Lys Ala	
	110 115	
	TGGCTGCCCG TTCGGGCAGC CACATCTCCC CATGACTCGA CGCTACGGGC TAGTCCCCGC	804
	CTCGGGTGTG GGTGAAGGAG AGCAC ATG AGC CAA CCA TCT TAT GGC CCG CTG	856
35	Met Ser Gln Pro Ser Tyr Gly Pro Leu	
	1 5	
	TTC GAG GCC CTG GCC CAC TAC AAT GAC AAG CTG CTG GCC ATG GCC AAG	904
	Phe Glu Ala Leu Ala His Tyr Asn Asp Lys Leu Leu Ala Met Ala Lys	
	10 15 20 25	
40	GCC CAG ACA GAG CGC ACC GCC CAG GCG CTG CTG CAG ACC AAT CTG GAC	952
	Ala Gln Thr Glu Arg Thr Ala Gln Ala Leu Leu Gln Thr Asn Leu Asp	
	30 35 40	
	GAT CTG GGC CAG GTG CTG GAG CAG GGC AGC CAG CAA CCC TGG CAG CTG	1000
	Asp Leu Gly Gln Val Leu Glu Gln Gly Ser Gln Gln Pro Trp Gln Leu	
	45 50 55	
	ATC CAG GCC CAG ATG AAC TGG TGG CAG GAT CAG CTC AAG CTG ATG CAG	1048
	Ile Gln Ala Gln Met Asn Trp Trp Gln Asp Gln Leu Lys Leu Met Gln	
45	60 65 70	
	CAC ACC CTG CTC AAA AGC GCA GGC CAG CCG AGC GAG CCG GTG ATC ACC	1096
	His Thr Leu Leu Lys Ser Ala Gly Gln Pro Ser Glu Pro Val Ile Thr	
	75 80 85	
	CCG GAG CGC AGC GAT CGC CGC TTC AAG GCC GAG GCC TGG AGC GAA CAA	1144
	Pro Glu Arg Ser Asp Arg Phe Lys Ala Glu Ala Trp Ser Glu Gln	
50	90 95 100 105	
	CCC ATC TAT GAC TAC CTC AAG CAG TCC TAC CTG CTC ACC GCC AGG CAC	1192
	Pro Ile Tyr Asp Tyr Leu Lys Gln Ser Tyr Leu Leu Thr Ala Arg His	
	110 115 120	
	CTG CTG GCC TCG GTG GAT GCC CTG GAG GGC GTC CCC CAG AAG AGC CGG	1240

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EP 0 824 148 A2

	Leu	Leu	Ala	Ser	Val	Asp	Ala	Leu	Glu	Gly	Val	Pro	Gln	Lys	Ser	Arg	
				125					130					135			
	GAG	CGG	CTG	CGT	TTC	TTC	ACC	CGC	CAG	TAC	GTC	AAC	GCC	ATG	GCC	CCC	1288
5	Glu	Arg	Leu	Arg	Phe	Phe	Thr	Arg	Gln	Tyr	Val	Asn	Ala	Met	Ala	Pro	
				140					145					150			
	AGC	AAC	TTC	CTG	GCC	ACC	AAC	CCC	GAG	CTG	CTC	AAG	CTG	ACC	CTG	GAG	1336
	Ser	Asn	Phe	Leu	Ala	Thr	Asn	Pro	Glu	Leu	Leu	Lys	Leu	Thr	Leu	Glu	
				155					160					165			
	TCC	GAC	GGC	CAG	AAC	CTG	GTG	CGC	GGA	CTG	GCC	CTC	TTG	GCC	GAG	GAT	1384
10	Ser	Asp	Gly	Gln	Asn	Leu	Val	Arg	Gly	Leu	Ala	Leu	Leu	Ala	Glu	Asp	
				170					175					180		185	
	CTG	GAG	CGC	AGC	GCC	GAT	CAG	CTC	AAC	ATC	CGC	CTG	ACC	GAC	GAA	TCC	1432
	Leu	Glu	Arg	Ser	Ala	Asp	Gln	Leu	Asn	Ile	Arg	Leu	Thr	Asp	Glu	Ser	
				190											200		
	GCC	TTC	GAG	CTC	GGG	CGG	GAT	CTG	GCC	CTG	ACC	CCG	GGC	CGG	GTG	GTG	1480
	Ala	Phe	Glu	Leu	Gly	Arg	Asp	Leu	Ala	Leu	Thr	Pro	Gly	Arg	Val	Val	
15				205						210					215		
	CAG	CGC	ACC	GAG	CTC	TAT	GAG	CTC	ATT	CAG	TAC	AGC	CCG	ACT	ACC	GAG	1528
	Gln	Arg	Thr	Glu	Leu	Tyr	Glu	Leu	Ile	Gln	Tyr	Ser	Pro	Thr	Thr	Glu	
				220					225					230			
	ACG	GTG	GGC	AAG	ACA	CCT	GTG	CTG	ATA	GTG	CCG	CCC	TTC	ATC	AAC	AAG	1576
	Thr	Val	Gly	Lys	Thr	Pro	Val	Leu	Ile	Val	Pro	Pro	Phe	Ile	Asn	Lys	
20				235					240					245			
	TAC	TAC	ATC	ATG	GAC	ATG	CGG	CCC	CAG	AAC	TCC	CTG	GTC	GCC	TGG	CTG	1624
	Tyr	Tyr	Ile	Met	Asp	Met	Arg	Pro	Gln	Asn	Ser	Leu	Val	Ala	Trp	Leu	
				250					255					260		265	
	GTC	GCC	CAG	GGC	CAG	ACG	GTA	TTC	ATG	ATC	TCC	TGG	CGC	AAC	CCG	GGC	1672
	Val	Ala	Gln	Gly	Gln	Thr	Val	Phe	Met	Ile	Ser	Trp	Arg	Asn	Pro	Gly	
				270										275		280	
25	GTG	GCC	CAG	GCC	CAA	ATC	GAT	CTC	GAC	GAC	TAC	GTG	GTG	GAT	GGC	GTC	1720
	Val	Ala	Gln	Ala	Gln	Ile	Asp	Leu	Asp	Asp	Tyr	Val	Val	Asp	Gly	Val	
				285						290					295		
	ATC	GCC	GCC	CTG	GAC	GGC	GTG	GAG	GCG	GCC	ACC	GGC	GAG	CGG	GAG	GTG	1768
	Ile	Ala	Ala	Leu	Asp	Gly	Val	Glu	Ala	Ala	Thr	Gly	Glu	Arg	Glu	Val	
				300					305					310			
30	CAC	GGC	ATC	GGC	TAC	TGC	ATC	GGC	GGC	ACC	GCC	CTG	TCG	CTC	GCC	ATG	1816
	His	Gly	Ile	Gly	Tyr	Cys	Ile	Gly	Gly	Thr	Ala	Leu	Ser	Leu	Ala	Met	
				315					320					325			
	GGC	TGG	CTG	GCG	GCG	CGG	CGC	CAG	AAG	CAG	CGG	GTG	CGC	ACC	GCC	ACC	1864
	Gly	Trp	Leu	Ala	Ala	Arg	Arg	Gln	Lys	Gln	Arg	Val	Arg	Thr	Ala	Thr	
				330					335					340		345	
35	CTG	TTC	ACT	ACC	CTG	CTG	GAC	TTC	TCC	CAG	CCC	GGG	GAG	CTT	GGC	ATC	1912
	Leu	Phe	Thr	Thr	Leu	Asp	Phe	Ser	Gln	Pro	Gly	Glu	Leu	Gly	Ile		
				350						355					360		
	TTC	ATC	CAC	GAG	CCC	ATC	ATA	GCG	GCG	CTC	GAG	GCG	CAA	AAT	GAG	GCC	1960
	Phe	Ile	His	Glu	Pro	Ile	Ile	Ala	Ala	Leu	Glu	Ala	Gln	Asn	Glu	Ala	
				365					370					375			
40	AAG	GGC	ATC	ATG	GAC	GGG	CGC	CAG	CTG	GCG	GTC	TCC	TTC	AGC	CTG	CTG	2008
	Lys	Gly	Ile	Met	Asp	Gly	Arg	Gln	Leu	Ala	Val	Ser	Phe	Ser	Leu	Leu	
				380					385					390			
	CGG	GAG	AAC	AGC	CTC	TAC	TGG	AAC	TAC	TAC	ATC	GAC	AGC	TAC	CTC	AAG	2056
	Arg	Glu	Asn	Ser	Leu	Tyr	Trp	Asn	Tyr	Tyr	Ile	Asp	Ser	Tyr	Leu	Lys	
				395					400					405			
45	GGT	CAG	AGC	CCG	GTG	GCC	TTC	GAT	CTG	CTG	CAC	TGG	AAC	AGC	GAC	AGC	2104
	Gly	Gln	Ser	Pro	Val	Ala	Phe	Asp	Leu	Leu	His	Trp	Asn	Ser	Asp	Ser	
				410					415					420		425	
	ACC	AAT	GTG	GCG	GGC	AAG	ACC	CAC	AAC	AGC	CTG	CTG	CGC	CGT	CTC	TAC	2152
	Thr	Asn	Val	Ala	Gly	Lys	Thr	His	Asn	Ser	Leu	Leu	Arg	Arg	Leu	Tyr	
				430						435					440		
50	CTG	GAG	AAC	CAG	CTG	GTG	AAG	GGG	GAG	CTC	AAG	ATC	CGC	AAC	ACC	CGC	2200
	Leu	Glu	Asn	Gln	Leu	Val	Lys	Gly	Glu	Leu	Lys	Ile	Arg	Asn	Thr	Arg	
				445					450					455			
	ATC	GAT	CTC	GGC	AAG	GTG	AAG	ACC	CCT	GTG	CTG	CTG	GTG	TCG	GCG	GTG	2248
	Ile	Asp	Leu	Gly	Lys	Val	Lys	Thr	Pro	Val	Leu	Leu	Val	Ser	Ala	Val	
				460					465					470			

55

GAC GAT CAC ATC GCC CTC TGG CAG GGC ACC TGG CAG GGC ATG AAG CTG 2296
 Asp Asp His Ile Ala Leu Trp Gln Gly Thr Trp Gln Gly Met Lys Leu
 475 480 485
 TTT GGC GGC GAG CAG CGC TTC CTC CTG GCG GAG TCC GGC CAC ATC GCC 2344
 Phe Gly Gly Glu Gln Arg Phe Leu Leu Ala Glu Ser Gly His Ile Ala
 490 495 500 505
 GGC ATC ATC AAC CCG CCG GCC GCC AAC AAG TAC GGC TTC TGG CAC AAC 2392
 Gly Ile Ile Asn Pro Pro Ala Ala Asn Lys Tyr Gly Phe Trp His Asn
 510 515 520
 GGG GCC GAG GCC GAG AGC CCG GAG AGC TGG CTG GCA GGG GCG ACG CAC 2440
 Gly Ala Glu Ala Glu Ser Pro Glu Ser Trp Leu Ala Gly Ala Thr His
 525 530 535
 CAG GGC GGC TCC TGG TGG CCC GAG ATG GGC TTT ATC CAG AAC CGT 2488
 Gln Gly Gly Ser Trp Trp Pro Glu Met Met Gly Phe Ile Gln Asn Arg
 540 545 550
 GAC GAA GGG TCA GAG CCC GTC CCC GCG CGG GTC CCG GAG GAA GGG CTG 2536
 Asp Glu Gly Ser Glu Pro Val Pro Ala Arg Val Pro Glu Glu Gly Leu
 555 560 565
 GCC CCC GCC CCC GGC CAC TAT GTC AAG GTG CCG CTC AAC CCC GTG TTT 2584
 Ala Pro Ala Pro Gly His Tyr Val Lys Val Arg Leu Asn Pro Val Phe
 570 575 580 585
 GCC TGC CCA ACA GAG GAG GAC GCC GCA TGAGCGCACA ATCCCTGGAA 2631
 Ala Cys Pro Thr Glu Glu Asp Ala Ala
 590
 GTAGGCCAGA AGGCCCGTCT CAGCAAGCGG TTCGGGGCGG CGGAGGTAGC CGCCTTCGCC 2691
 GCGCTCTCGG AGGACTTCAA CCCCCTGCAC CTGGACCCGG CCTTCGCCCG CACCACGGCG 2751
 TTCGAGCGGC CCATAGTCCA CGGCATGCTG CTCGCCAGCC TCTTCTCCGG GCTGCTGGGC 2811
 CAGCAGTTGC CGGGCAAGGG GAGCATCTAT CTGGGTCAAA GCCTCAGCTT CAAGCTGCCG 2871
 GTCTTTGTGC GGGACGAGGT GACGGCCGAG GTGGAGGTGA CCGCCCTTCG CGAGGACAAG 2931
 CCCATCGCCA CCCTGACCAC CCGCATCTTC ACCCAAGGCG GCGCCCTCGC CGTGACGGGG 2991
 GAAGCCGTGG TCAAGCTGCC TTAAGCACCG GCGGCACGCA GGCACAATCA GCCCGGCCCC 3051
 TGCCGGGCTG ATTGTTCTCC CCGCTCCGC TTGCCCCCTT TTCGGGGCA ATTTGGCCCA 3111
 GGCCCTTTCC CTGCCCGGCC TAACTGCCTA AAATGGCCGC CCTGCCGTGT AGGCATTCTAT 3171
 CCAGCTAGAG GAATTC 3187

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3187 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 2611..3012

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGATCTGGAC CGGGGTGCTG GCCTGGGCCA CGCCGGCGAG GGCCAGCGCG GAGCAACCGA 60
 GCAGCAGGGC GAGAGGTTTC ATCGGGATTC CTTGGCAGTC TGAATGACGT GCCAGCCTAT 120
 CAGCGCGGCG CCGGTGCGGC GAGGGCGCGC CGGACCCAGT GCGTCACCTC TCGTCTGATC 180
 CGCCTCCCTC GACGGGCGTC GCTGACAAAA AAATTCAAAC AGAAATTAAC ATTTATGTCA 240
 TTTACACCAA ACCGCATTTC GTTGCGAAT GCTCAAACGT GTGTTTGAAC AGAGCAAGCA 300
 ACACGTAAAC AGGGATGACA TGCAGTACCC GTAAGAAGGG CCGATTGGCC CACAACAACA 360
 CTGTTCTGCC GAACTGGAGA CCGATGATGA ATATGGACGT GATCAAGAGC TTTACCGAGC 420
 AGATGCAAGG CTTCCGCGCC CCCCTCAGCC GCTACAACCA GCTGCTGGCC AGCAACATCG 480
 AACAGCTGAC CCGGTTGCAG CTGGCCTCCG CCAACGCCTA CGCCGAAGTG GGCCTCAACC 540
 AGTTGCAGGC CGTGAGCAAG GTGCAGGACA CCCAGAGCCT GCGGGCCCTG GGCACAGTGC 600
 AACTGGAGAC CGCCAGCCAG CTCTCCCGCC AGATGCTGGA TGACATCCAG AAGCTGAGCG 660

	CCCTCGGCCA	GCAGTTCAAG	GAAGAGCTGG	ATGTCCTGAC	CGCAGACGGC	ATCAAGAAAA	720
	GCACGGGCAA	GGCCTGATAA	CCCCTGGCTG	CCCGTTCCGG	CAGCCACATC	TCCCCATGAC	780
5	TCGACGCTAC	GGGCTAGTTC	CCGCCTCGGG	TGTGGGTGAA	GGAGAGCACA	TGAGCCAACC	840
	ATCTTATGGC	CCGCTGTTTC	AGGCCCTGGC	CCACTACAAT	GACAAGCTGC	TGGCCATGSC	900
	CAAGGCCAG	ACAGAGCGCA	CCGCCCAGGC	GCTGCTGCAG	ACCAATCTGG	ACGATCTGGG	960
	CCAGGTGCTG	GAGCAGGGCA	GCCAGCAACC	CTGGCAGCTG	ATCCAGGCCC	AGATGAAGCTG	1020
	GTGGCAGGAT	CAGCTCAAGC	TGATGCAGCA	CACCCTGCTC	AAAAGGCGCAG	GCCAGCCGAG	1080
	CGAGCCGGTG	ATCACCCCGG	AGCGCAGCGA	TCGCCGCTTC	AAGGCCGAGG	CCTGGAGCGA	1140
	ACAACCCATC	TATGACTACC	TCAAGCAGTC	CTACCTGCTC	ACCGCCAGGC	ACCTGCTGGC	1200
10	CTCGGTGGAT	GCCCTGGAGG	GCGTCCCCCA	GAAGAGCCCG	GAGCGGCTGC	GTTTCTTCAC	1260
	CCGCCAGTAC	GTCAACGCCA	TGGCCCCCAG	CAACTTCCTG	GCCACCAACC	CCGAGCTGCT	1320
	CAAGCTGACC	CTGGAGTCCG	ACGGCCAGAA	CCTGGTGGCG	GGACTGGCCC	TCTTGGCCGA	1380
	GGATCTGGAG	CGCAGCGCCG	ATCAGCTCAA	CATCCGCTG	ACCGACGAAT	CCGCCTTCGA	1440
	GCTCGGGCGG	GATCTGGCCC	TGACCCCGGG	CCGGGTGGTG	CAGCGCACCG	AGCTCTATGA	1500
	GCTCATTAC	TACAGCCCGA	CTACCGAGAC	GGTGGGCAAG	ACACCTGTGC	TGATAGTGCC	1560
15	GCCCTTCATC	AACAAGTACT	ACATCATGGA	CATGCGGCCC	CAGAACTCCC	TGGTGGCCTG	1620
	GCTGGTCGCC	CAGGGCCAGA	CGGTATTAT	GATCTCCTGG	CGCAACCCCG	GCGTGGCCCA	1680
	GGCCCCAATC	GATCTCGACG	ACTACGTGGT	GGATGGCGTC	ATCGCCGCC	TGGACGGCGT	1740
	GGAGGCGGCC	ACCGGCGAGC	GGGAGGTGCA	CGGCATCGGC	TACTGCATCG	GCGGCACCGC	1800
	CCTGTCGCTC	GCCATGGGCT	GGCTGGCGGC	GCGGCGCCAG	AAGCAGCGGG	TGCGCACCGC	1860
	CACCCTGTTC	ACTACCCTGC	TGGACTTCTC	CCAGCCCGGG	GAGCTTGGCA	TCTTCATCCA	1920
20	CGAGCCCATC	ATAGCGGCGC	TCGAGGCGCA	AAATGAGGCC	AAGGGCATCA	TGGACGGGCG	1980
	CCAGCTGGCG	GTCTCCTTCA	GCCTGTGCG	GGAGAACAGC	CTCTACTGGA	ACTACTACAT	2040
	CGACAGCTAC	CTCAAGGGTC	AGAGCCCGGT	GGCCTTCGAT	CTGTGCACT	GGAACAGCGA	2100
	CAGCACCAAT	GTGGCGGGCA	AGACCCACAA	CAGCCTGCTG	CGCCGTCTCT	ACCTGGAGAA	2160
	CCAGCTGGTG	AAGGGGGAGC	TCAAGATCCG	CAACACCCGC	ATCGATCTCG	GCAAGGTGAA	2220
	GACCCCTGTG	CTGCTGGTGT	CGGCGGTGGA	CGATCACATC	GCCCTCTGGC	AGGGCACCTG	2280
	GCAGGGCATG	AAGCTGTTTG	GCGGGGAGCA	GCGCTTCCTC	CTGGCGGAGT	CCGGCCACAT	2340
25	GCGCGGCATC	ATCAACCCGC	CGGCCGCCAA	CAAGTACGGC	TTCTGGCACA	ACGGGGCCGA	2400
	GGCCGAGAGC	CCGGAGAGCT	GGCTGGCAGG	GCGACGACAC	CAGGGCGGCT	CCTGGTGGCC	2460
	CGAGATGATG	GGCTTTATCC	AGAACCGTGA	CGAAGGGTCA	GAGCCCGTCC	CCGCGCGGGT	2520
	CCCGGAGGAA	GGGTGGCCCC	CCGCCCCCGG	CCACTATGTC	AAGGTGCGGC	TCAACCCCGT	2580
	GTTTGCCTGC	CCAACAGAGG	AGGACGCCGC	ATG AGC GCA CAA TCC CTG GAA GTA			2634
				Met Ser Ala Gln Ser Leu Glu Val			
30				1	5		
	GGC CAG AAG GCC CGT CTC AGC AAG CGG TTC GGG GCG GCG GAG GTA GCC						2682
	Gly Gln Lys Ala Arg Leu Ser Lys Arg Phe Gly Ala Ala Glu Val Ala						
	10	15	20				
	GCC TTC GCC GCG CTC TCG GAG GAC TTC AAC CCC CTG CAC CTG GAC CCG						2730
	Ala Phe Ala Ala Leu Ser Glu Asp Phe Asn Pro Leu His Leu Asp Pro						
	25	30	35	40			
35	GCC TTC GCC GCC ACC ACG GCG TTC GAG CGG CCC ATA GTC CAC GGC ATG						2778
	Ala Phe Ala Ala Thr Thr Ala Phe Glu Arg Pro Ile Val His Gly Met						
	45	50	55				
	CTG CTC GCC AGC CTC TTC TCC GGG CTG CTG GGC CAG CAG TTG CCG GGC						2826
	Leu Leu Ala Ser Leu Phe Ser Gly Leu Leu Gly Gln Gln Leu Pro Gly						
	60	65	70				
40	AAG GGG AGC ATC TAT CTG GGT CAA AGC CTC AGC TTC AAG CTG CCG GTC						2874
	Lys Gly Ser Ile Tyr Leu Gly Gln Ser Leu Ser Phe Lys Leu Pro Val						
	75	80	85				
	TTT GTC GGG GAC GAG GTG ACG GCC GAG GTG GAG GTG ACC GCC CTT CGC						2922
	Phe Val Gly Asp Glu Val Thr Ala Glu Val Glu Val Thr Ala Leu Arg						
	90	95	100				
45	GAG GAC AAG CCC ATC GCC ACC CTG ACC ACC CGC ATC TTC ACC CAA GGC						2970
	Glu Asp Lys Pro Ile Ala Thr Leu Thr Thr Arg Ile Phe Thr Gln Gly						
	105	110	115	120			
	GGC GCC CTC GCC GTG ACG GGG GAA GCC GTG GTC AAG CTG CCT						3012
	Gly Ala Leu Ala Val Thr Gly Glu Ala Val Val Lys Leu Pro						
	125	130					
50	TAAGCACCGG CGGCACGAG GCACAATCAG CCCGGCCCCT GCCGGGCTGA TTGTTCTCCC						3072
	CCGCTCCGCT TGCCCCCTTT TTCGGGGCAA TTTGGCCCAG GCCCTTTPCC TGCCCCGCT						3132
	AACTGCCTAA AATGGCCGCC CTGCCGTGTA GGCATTATC CAGCTAGAGG AATTC						3187

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AGTTCCCGCC TCGGGTGTGG GTGAA

- (2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGCATATGCG CTCATGCGGC GTCCT 25

- (2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GCCATATGAG CGCACAAATCC CTGGAAGTAG

- (2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CTGGGATCCG CCGGTGCTTA AGGCAGCTTG

- (2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Ser Ala Gln Ser Leu Glu Val Gly Gln Lys Ala Arg Leu Ser Lys Arg
 1 5 10 15
 Phe Gly Ala Ala
 20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

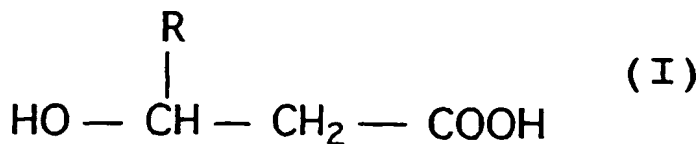
Met Ser Ala Gln Ser Leu Glu Val Gly Gln Lys Ala Arg Leu Ser Lys
 1 5 10 15
 Arg Phe Gly Ala Ala
 20

Claims

1. A polyester synthase gene coding for a polypeptide containing the amino acid sequence of SEQ ID NO:2 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about polyester synthase activity.
2. A polyester synthase gene comprising the nucleotide sequence of SEQ ID NO:1.
3. A gene expression cassette comprising the polyester synthase gene of claims 1 or 2 and either of open reading frames located upstream and downstream of said gene.
4. The gene expression cassette according to claim 3, wherein the open reading frame located upstream of the polyester synthase gene comprises DNA coding for the amino acid sequence of SEQ ID NO:4.
5. The gene expression cassette according to claim 3, wherein the open reading frame located upstream of the polyester synthase gene comprises the nucleotide sequence of SEQ ID NO:3.
6. The gene expression cassette according to claim 3, wherein the open reading frame located downstream of the polyester synthase gene comprises DNA coding for a polypeptide containing the amino acid sequence of SEQ ID NO:6 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about enoyl-CoA hydratase activity.
7. The gene expression cassette according to claim 3, wherein the open reading frame located downstream of the

polyester synthase gene comprises the nucleotide sequence of SEQ ID NO:5.

8. A recombinant vector comprising the polyester synthase gene of claim 1 or 2 or the gene expression cassette of any one of claims 3 to 7.
9. A transformant transformed with the recombinant vector of claim 8.
10. A process for producing polyester, wherein the transformant of claim 9 is cultured in a medium and polyester is recovered from the resulting culture.
11. The process for producing polyester according to claim 10, wherein the polyester is a copolymer of 3-hydroxyalkanoic acid represented by formula I:



wherein R represents a hydrogen atom or a C1 to C4 alkyl group.

12. The process for producing polyester according to claim 10, wherein the polyester is a poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) random copolymer.

FIG. 1A

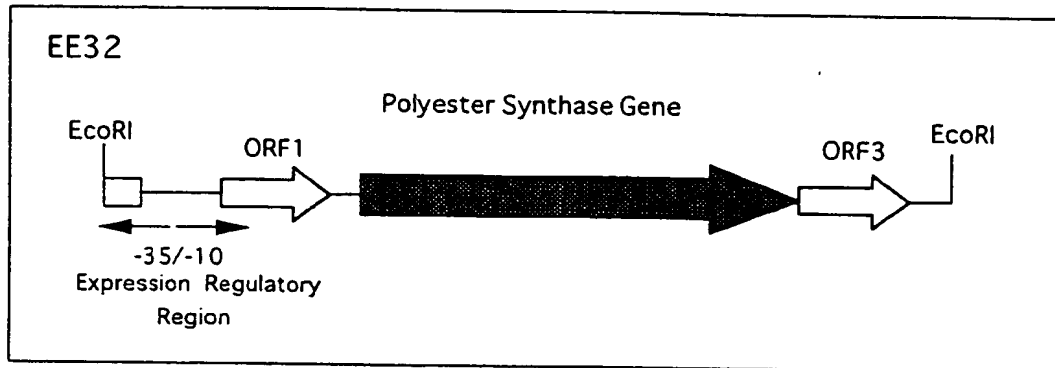


FIG. 1B



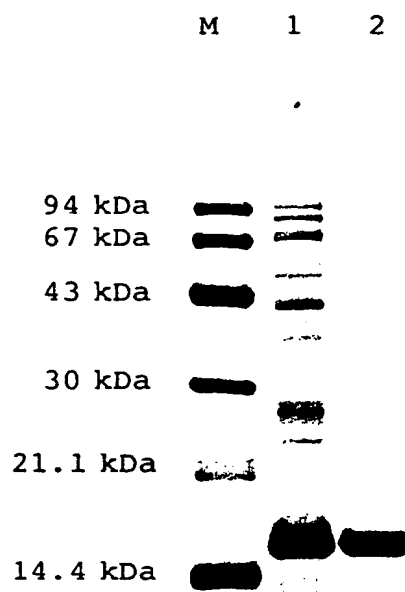
FIG. 1C



FIG. 1D



FIG.2



Lane M: molecular-weight marker

Lane 1: soluble-protein fraction from NB3

Lane 2: active fraction eluted from the anion
exchange column

(19)



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(54) Polyester synthase gene and process for producing polyester

(57) The present invention relates to a polyester synthase gene coding for a polypeptide containing the amino acid sequence of SEQ ID NO:2 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about polyester synthase activity; a gene expression cassette comprising the polyester synthase gene and either of open reading frames located upstream and downstream of said gene; a recombinant vector comprising the gene expression cassette; a transformant transformed with the recombinant vector; and a process for producing polyester by culturing the transformant in a medium and recovering polyester from the resulting culture.

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EUROPEAN SEARCH REPORT

Application Number
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Place of search THE HAGUE		Date of completion of the search 25 August 1999	Examiner Delanghe, L
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